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Circulating Factors That Modify Lung Cell DNA Synthesis Following Exposure to Inhaled Oxidants. II. Effect of Serum and Lavage on Lung Pneumocytes Following Exposure of Adult Rats to 1 PPM Ozone

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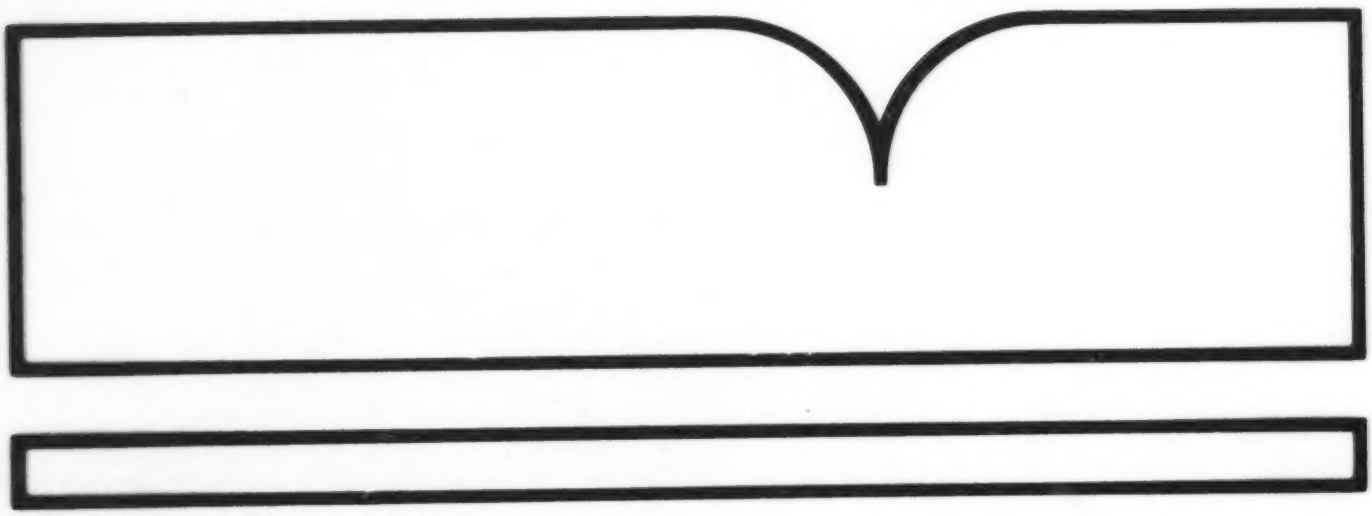

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16. ABSTRACT

Adult rats were exposed to 1 ppm (1.96 mg/m^3) ozone or air for 2 weeks. Animals were sacrificed at 3,5,7 or 14 days after the onset of exposure and samples of plasma and lung lavage obtained. Heat inactivated plasma and lavage from animals exposed to ozone, for 5 or 7 days, significantly increased DNA synthesis by lung fibroflasts compared with plasma or lavage from air exposed animals. Fractionation of plasma and lavage samples indicated that the factor responsible had an isoelectric point of 6.45-6.75, and a molecular weight of 38±3 Kd. This factor has a dose-dependent effect on lung pneumocyte DNA synthesis in culture. It has no effect on cultured fibroblast DNA synthesis, and is distinct from a previously described factor in the plasma of these ozone-exposed animals which enhances fibroblast DNA synthesis. The factor is detectable within 5 days of exposure, and may hold some promise as a marker of early oxidant lung injury.

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CIRCULATING FACTORS THAT MODIFY LUNG CELL DNA SYNTHESIS FOLLOWING EXPOSURE TO INHALED OXIDANTS. II. EFFECT OF SERUM AND LAVAGE ON LUNG PNEUMOCYTES FOLLOWING EXPOSURE OF ADULT RATS TO 1 PPM OZONE

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Adult rats were exposed to 1 ppm (1.96 mg/m^3) ozone or air for 2 wk. Animals were sacrificed at 3, 5, 7, or 14 d after the onset of exposure, and samples of plasma and lung lavage were obtained. Heat-inactivated plasma and lavage from animals exposed to ozone for 5 or 7 d significantly increased DNA synthesis by lung pneumocytes compared with plasma or lavage from air-exposed animals. Fractionation of plasma

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The research described in this article has been reviewed by the Health Effects Research Laboratory, U.S. Environmental Protection Agency, and approved for publication. Approval does not signify that the contents necessarily reflect the views and policies of the agency, nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

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and lavage samples indicated that the factor responsible had an isoelectric point of 6.45–6.75, and a molecular weight of 38 ± 3 kDa. This factor has a dose-dependent effect on lung pneumocyte DNA synthesis in culture. It has no effect on cultured fibroblast DNA synthesis, and is distinct from a previously described factor in the plasma of these ozone-exposed animals that enhances fibroblast DNA synthesis. The factor is detectable within 5 d of exposure, and may hold some promise as a marker of early oxidant lung injury.

INTRODUCTION

Inhalation of sublethal concentrations of both O_2 and O_3 results in type I pneumocyte destruction, followed by type II pneumocyte hyperplasia and interstitial thickening, with an increase of fibroblasts (Crapo et al., 1984; Evans, 1984). We speculate that since both pneumocyte and fibroblast hyperplasias are early events in oxidant lung injury, detection of any circulating growth factors that control such cellular changes could serve as a marker of early oxidant lung injury.

There is experimental support for the presence of circulating factors from double intubation experiments (Motlagh et al., 1969) in which there was early release of factors from an ipsilateral oxygen-exposed lung that affected the air-exposed contralateral lung. To be of practical value, any such factors would have to be detectable in serum, rather than in lung lavage, to maintain a low level of invasiveness. The early increase in epithelial and endothelial layer permeability observed with O_3 exposure (Bhalla et al., 1987) suggests that even if the growth factors responsible for pulmonary cellular hyperplasia are autocrine or paracrine in nature, any water-soluble polypeptide growth factors would have access to the circulation, and should thus be detectable in the blood, with a sufficiently sensitive assay.

We have previously used an animal model, in which adult rats were exposed to 1 ppm (1.96 mg/m^3) ozone or air for 2 wk, to examine the hypothesis that factors that influence lung fibroblast growth appear in serum early in the course of lung oxidant injury with O_3 (Tanswell et al., 1989). Plasma from animals exposed to ozone for 7 or 14 d significantly increased DNA synthesis by lung fibroblasts compared with plasma from air-exposed animals. Fractionation of plasma and lavage samples indicated that the factor responsible had an isoelectric point of 6.45–6.75, and a molecular weight of 32 ± 2 kDa. This factor had a dose-dependent effect on lung fibroblast DNA synthesis in culture, but no significant effect on cultured pneumocyte DNA synthesis.

The same plasma and lavage samples used for analysis of effects on fibroblast DNA synthesis have been further examined for effect on pneumocyte DNA synthesis.

METHODS

Animal Exposures

All exposures were conducted with Charles River CD strain male rats of 400 ± 25 g at the onset of exposure. Ozone exposures were conducted at the Health Effects Laboratory of the Environmental Protection Agency, Research Triangle Park, N.C. Animals were exposed to either filtered air or 1 ppm O_3 (1.96 mg/mg^3) for up to 14 d, with sacrifice of 10 animals from each group for sample collection at 3, 5, 7, or 14 d from the onset of exposure. A detailed description of the exposure system was provided in an earlier report (Tanswell et al., 1989). The choice of a 14-d exposure period and the use of a 1 ppm concentration were based on morphologic data (Evans, 1984), demonstrating that both pneumocyte and fibroblast hyperplasias occur with these exposure conditions. The choice of 3, 5, and 7 d intermediate time points was arbitrary. Sacrifice was by halothane inhalation to cardiac arrest.

Sample Collection and Preparation

At sacrifice all animals were weighed and allocated a code number, which was used to identify collected samples of plasma and lung lavage. Blood was collected from the exposed abdominal aorta into a syringe (prechilled to 4°C) containing 3.8% sodium citrate, to give approximately 1 volume of citrate to 9 volumes of blood. This was centrifuged at 2000 × g for 15 min at 4°C, and the plasma was aspirated for recentrifugation at 22,000 × g for 60 min, to remove any residual red cell contamination, then recalcified with 20 mM calcium chloride. This material is subsequently referred to as plasma in the text, despite recalcification, because this was the form in which it was collected. The lungs were lavaged 3 consecutive times with 5 ml phosphate-buffered saline. The combined lavage fluids from each animal were centrifuged at 300 × g for 10 min at 4°C to remove contaminating cells. The final volumes of plasma and lavage were recorded with the animal's code number, and the samples were frozen at -70°C for storage in siliconized glassware prior to lyophilization. Siliconized glassware was used to prevent growth factor adherence to uncoated glass.

The plasma samples were resuspended to their original volume in distilled water, filtered twice through a 0.22-μm syringe filter, and diluted to 10% with culture medium for bioassay. The lavage samples were resuspended to an arbitrary volume of a 5 ml in distilled H_2O and dialyzed across a dialysis membrane, with a 3500 molecular weight exclusion limit, against an excess of distilled H_2O. The dialysis was conducted over 72 h at 4°C, with 3 changes of distilled H_2O. After dialysis the lavage was lyophilized and resuspended in 2 ml phosphate-buffered saline. The

samples were diluted to 10% with culture medium containing 2% fetal bovine serum (FBS) for bioassay.

These same samples were used in a previous study of growth factors for lung fibroblasts (Tanswell et al., 1989).

Bioassay

All samples were tested against purified preparations of fetal rat (20 d of gestation; term = 22 d) lung pneumocytes. Fetal cells were used for bioassay in preference to adult cells because adult type II pneumocytes do not normally divide in culture, and have very low rates of DNA synthesis. Also the fetal lung is obtained from a sterile environment within the amniotic sac, and culture loss or bioassay artefact due to contamination is infrequent.

Times gestation Sprague-Dawley rats were sacrificed on d 20 of gestation by inhalation of saturated chloroform vapors. Five litters were used for each preparation. The uterine horns were immediately exposed through an abdominal incision, and the fetuses were removed following intrauterine decapitation. The bodies were placed in calcium- and magnesium-free Hanks balanced salt solution (HBSS) at 4°C. The lungs were excised, dissected free of major airways etc., and pooled. A suspension of mixed lung cells was prepared as previously reported (Tanswell et al., 1989). Rat fetal lung pneumocytes were prepared using a minor modification of the method of Post et al. (1984). The filtered cell suspension was pelleted at 300 × g for 10 min. The pellet was resuspended in Eagle's minimal essential medium with Earle's salts, 50 μg/ml gentamycin, 2.5 μg/ml amphotericin B, and 1% nonessential amino acids containing 10% fetal bovine serum and plated in a 25-cm^2 culture flask for 60 min at 37°C. During this time lung fibroblasts, but few other lung cells, attach. The nonadherent cells were removed and pelleted, then allowed to stand for 60 min at 37°C before being resuspended in 1.3 ml fresh medium with 10% serum. This cell suspension was applied as 50-μl aliquots to 24 1-cm^3 gelatin sponges saturated with medium with 10% serum. After 1 h the culture flasks containing the sponges (8/flask) had culture medium added to float the sponges, which were then left to incubate at 37°C overnight. Organotypic cultures prepared in this way contain primarily pneumocytes with some residual fibroblast contamination (Simpson et al., 1985). The gelatin sponges were dissolved with 1% collagenase in Hanks balanced salt solution. Following the addition of serum to 10% by volume, the released cells were pelleted at 300 × g for 10 min. These were resuspended in fresh medium with 10% serum and incubated at 37°C for 15 min to allow any residual fibroblasts to attach. These fibroblasts were retained in culture for use as negative controls. Nonadherent cells were plated in medium with 10% serum and grown to confluence. The flask was split 1:3 with 0.05% trypsin/0.02% EDTA and replated following another 15-min differential adherence step. These cells were

grown to confluence in the second passage with medium, in which L-valine was replaced by D-valine to inhibit fibroblast growth (Sordillo and Oliver, 1988), and 10% serum. The type II pneumocytes were then removed from the flasks with 0.05% trypsin/0.02% EDTA for use in the bioassay. Purity of the type II cell population at the time of assay is > 95%, as assessed by indirect immunofluorescent staining for surfactant apoproteins (Post and Smith, 1988), and by light microscopy following tannic acid and polychrome staining for lamellar body identification (Mason, 1985).

Effect of Sample Addition on Lung Cell DNA Synthesis

The effect of sample addition on DNA synthesis by isolated fetal rat lung pneumocytes was assessed by incorporation of [^{3}H]thymidine into DNA by a modification of the method of Greenstein et al. (1984), as previously described with fibroblasts (Tanswell et al. 1989).

Each sample (plasma or lavage diluted as described above) from the 10 animals at each time point for each of the 4 exposure groups was assayed against pneumocytes as an initial screen for activity. Plasma samples were also tested after dialysis across a membrane with a molecular weight exclusion limit of 3500 and heat treatment at 56°C for 30 min. This choice of temperature and time for heat inactivation of the plasma was based on the observation (Miyazaki et al., 1985) that a cell growth inhibitor, normally present in rat serum, can be inhibited by such treatment. The use of both crude and treated plasma samples was intended to overcome problems with dialyzable and heat-labile cell growth inhibitors normally present in adult rat serum, without missing potentially heat-labile or very low molecular weight mitogens present in the samples.

For the purposes of presentation, the [^{3}H]thymidine incorporation into DNA for each sample has been expressed as a percentage of the incorporation (cpm) into DNA by control cells to which no sample, only an equal volume of suspension buffer, had been added. Because there were no observed differences in DNA synthesis in any of our studies between the various air exposure groups, a combined result is presented.

Preparative Electrofocusing

An an initial screening procedure, aliquots of resuspended plasma samples (100 μl) and lavage samples (50 μl) for animals exposed to each gas for 3, 5 + 7, and 14 d were pooled. The pooled materials were dialyzed against 3 changes of an excess of 1% glycine for 72 h at 4°C. After reconstitution in an ampholine solution (pH 4–11), the samples were subjected to flat-bed electrophoresis in a granulated gel, as described in detail previously (Tanswell et al. 1989).

At the end of the separation period, a fractionating grid was pressed into the slurry and each of the 30 compartments was collected separately

with a spatula into disposable minicolumns. Each column was washed overnight with 2 ml of phosphate-buffered saline at pH 7.0, and the eluate was dialyzed against deionized distilled H_2O and lyophilized. Fractions were then taken up in 2 ml (plasma) or 800 μl (lavage) of culture medium. These volumes were diluted 10-fold for the bioassay. Each fraction of each sample was tested in triplicate for [^{3}H]thymidine incorporation into DNA.

The linearity of the pH gradient obtained in this system has been reported previously. All plots include a fourth-order polynomial regression line (r = .998) showing standard ampholine separation.

High-Performance Liquid Chromatography

Preparative electrofocusing fractions 12–16 from each exposure group were then subjected to a further separation by size using a Waters (Milford, Mass.) M600 high-performance liquid chromatography system with a Protein Pak 300SW column and a Protein Pak 125 column in series, as described previously (Tanswell et al., 1989).

The linearity of separation obtained using this column series was determined using a variety of molecular weight standards, where the eluate was monitored by absorbence at 280 nm. A third-order polynomial regression analysis was conducted by the method of least squares, and this regression line (r = .995) has been included in all data plots.

Sequential Electrofocusing and HPLC

The electrofocusing and HPLC studies above were all conducted on pooled material as a screening procedure to identify peaks of greatest interest. As a result of this screen it appeared that stimulatory activity for pneumocytes appeared in plasma following O_3 exposure, and that the factor responsible had an isoelectric point of 6.45–6.75 with an apparent molecular weight of 38 ± 3 kDa. To confirm these screening results, the original plasma samples were individually fractionated, first by preparative electrofocusing, then by HPLC, to isolate only those plasma components with the physical characteristics determined by the screening studies. The final material was reconstituted in culture medium to give dilutions of 5- or 10-fold relative to the original sample volume for assay, to determine if effects were dose-dependent. The 5-fold dilution material was also studied after the material had been heated at 56°C for 30 min.

The fivefold diluted material after heat treatment was also tested for effect against fetal lung fibroblasts.

Statistical Analysis

All values are shown as the mean ± standard deviation unless otherwise stated. Significance ($p < .05$) was determined by analysis if variance followed by both Dunnett's two-sided test and Duncan's multiple range test.

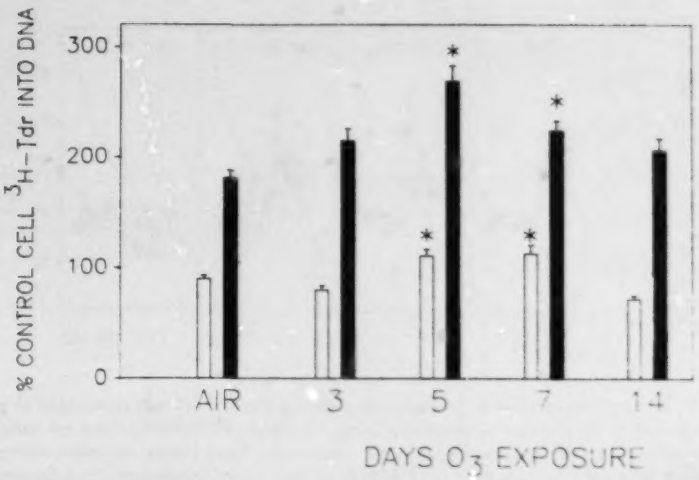

FIGURE 1. Effect of the addition of plasma and lung lavage from animals exposed to air or ozone on DNA synthesis by cultured lung pneumocytes. Plasma was used after heating and dialysis (■). Lavage was used after dialysis (□). Results are expressed as a percentage of the [^{3}H]thymidine (Tdr) incorporation into DNA observed in control cells not exposed to samples, only to suspension buffer; * $p < .05$ compared with the results for the comparable air group.

RESULTS

Ozone Exposure

The effect of ozone exposure on body weight, left lung weight, and left lung weight/body weight ratio $\times 10^3$ have been reported elsewhere (Tanswell et al., 1989). As would be expected, the animals exposed to air gained weight over the 2-wk exposure period, while those exposed to O_3 had a significantly reduced weight gain. The left lung weight did not change significantly over 2 wk for the air-exposed group. The O_3-exposed animals had significantly heavier lungs, and greater lung weight/body weight ratios, than the air controls as early as 3 d from the onset of exposure, which persisted throughout the exposure period. Visual inspection of the lungs on removal resulted in one animal from the air group being discarded from the analysis due to a blotchiness of the lung surface.

Initial Screening of Individual Samples

The result of the initial screening of all samples from the O_3 and air exposure against lung pneumocytes is shown in Fig. 1, with the air group containing samples from all 39 animals.

Crude plasma caused a profound inhibition of [^{3}H]thymidine incorporation into DNA irrespective of the group from which the plasma was

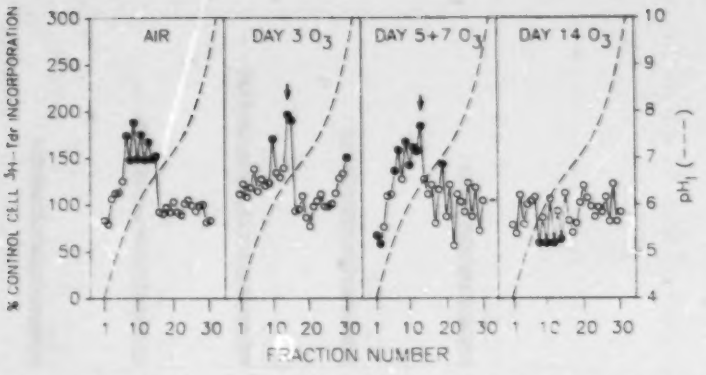

FIGURE 2. Effect of the addition of pooled plasma from groups of animals exposed to air or ozone, after fractionation by preparative electrofocusing, on DNA synthesis by cultured pneumocytes. Each plot point is the mean value for triplicate estimations. Solid circles represent values that are significantly different from values for [^{3}H]thymidine (Tdr) incorporation into DNA by control cells not exposed to sample fractions, only to suspension buffer. The arrows indicate the peak of interest. The interrupted line represents the standard curve for separation according to pH as shown on the right axis.

obtained (data not shown). This was reversed by dialysis and heat treatment of plasma samples.

Pneumocyte DNA synthesis was significantly increased in the presence of plasma or lavage from animals exposed to 1 ppm O_3 (1.96 mg/m^3) for 5 or 7 d when compared with plasma from air controls.

Electrofocusing of Pooled Samples

Alterations of pneumocyte DNA synthesis by fractionated plasma and lavage from air- and O_3-exposed animals are shown in Fig. 2 and 3, respectively. Control plasma had stimulatory activity in fractions 7–15. This profile was lost by 3 d of O_3-exposure, and was replaced by a narrow peak of stimulatory activity in fraction 15 ± 1 on d 3 and 5 + 7 of exposure. This activity was also present in lavage at d 3 of exposure.

HPLC of Pooled Samples

Preparative electrofocusing fractions 12–16 of plasma and lavage were further fractionated by HPLC. The effect of these fractions from d 5 + 7 lavage on pneumocyte DNA synthesis is shown in Fig. 4. Lavage from control animals exposed to air showed a narrow peak of stimulatory activity in fraction 31. This activity is markedly increased in the ozone-exposed animals, with greater activity being evident in fractions 28–34. Bioassay of HPLC fractions of plasma (Fig. 5) revealed several peaks of activity, with various molecular weights, in samples from ozone-exposed animals. No stimulatory activity was evident in samples from air-

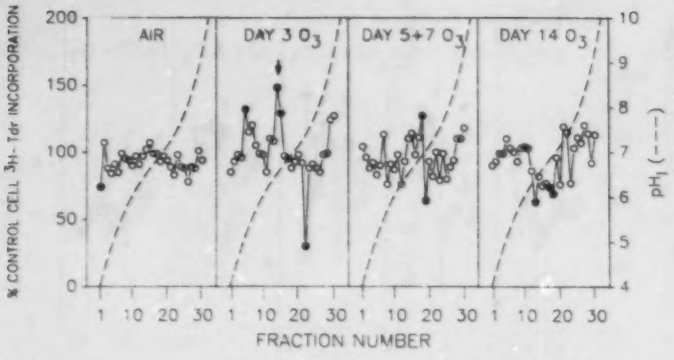

FIGURE 3. Effect of the addition of pooled lavage from groups of animals exposed to air or ozone, after fractionation by preparative electrofocusing, on DNA synthesis by cultured pneumocytes. Each plot point is the mean value for triplicate estimations. Solid circles represent values that are significantly different from values for [^{3}H]thymidine (Tdr) incorporation into DNA by control cells not exposed to sample fractions, only to suspension buffer. The arrow indicates the peak of interest. The interrupted line represents the standard curve for separation according to pH as shown on the right axis.

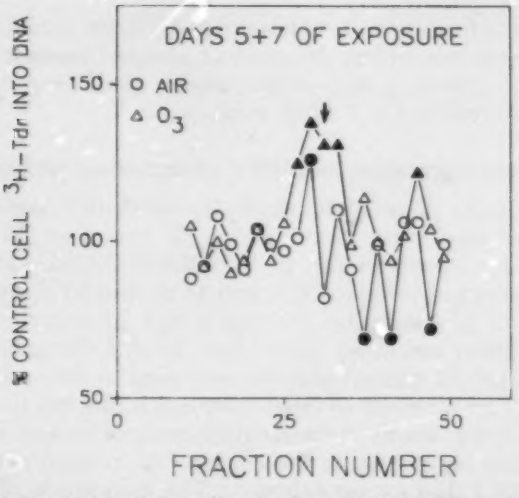

FIGURE 4. Effect of the addition of electrofocusing fractions 12–16 of lavage from groups of animals exposed to air (○) or ozone (△) for 5 and 7 d, after further separation by high-performance liquid chromatography, on DNA synthesis by pneumocytes. Each plot point is the mean value for triplicate estimations. Solid symbols represent values that are significantly different from values for [^{3}H]thymidine (Tdr) incorporation into DNA by control cells not exposed to sample fractions, only to suspension buffer. The arrows indicate the peak of interest.

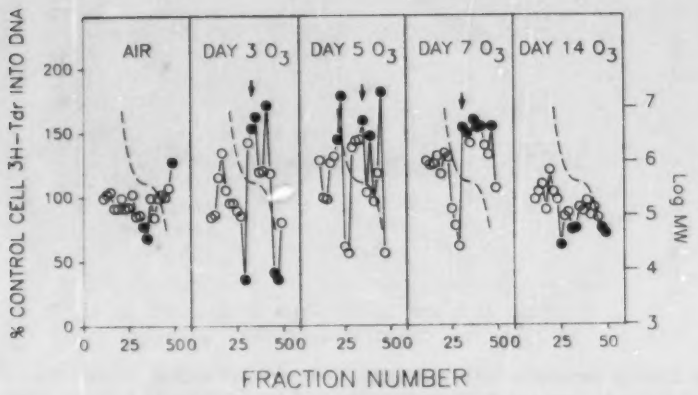

FIGURE 5. Effect of the addition of electrofocusing fractions 12–16 of plasma from groups of animals exposed to air or ozone, after further separation by high-performance liquid chromatography, on DNA synthesis by cultured pneumocytes. Each plot point is the mean value for triplicate estimations. Solid circles represent values that are significantly different from values for [^{3}H]thymidine (Tdr) incorporation into DNA by control cells not exposed to sample fractions, only to suspension buffer. The arrows indicate the peak of interest. The interrupted line represents the standard curve for separation by size as shown on the right axis.

exposed animals. Fractions 31 ± 3, with an apparent molecular weight of 38 ± 3 kDa, were selected as the peak of greatest interest based on the presence of stimulatory activity in this region also being evident in fractionated lavage from d 5 + 7 of O_3 exposure.

Sequential Electrofocusing and HPLC of Individual Samples

Bioassay results of original plasma samples that were sequentially fractionated by isoelectric point (6.45–6.75) and size (38 ± 3 kDa) on pneumocytes are shown in Fig. 6. The 10-fold diluted plasma samples from animals exposed to O_3 for 3, 7, and 14 d showed significantly stimulatory activity for pneumocytes compared with plasma from air exposed animals. This effect was dose dependent, in that these effects were reduced when fivefold diluted plasma was used in the assay, suggesting the presence of an inhibitor of DNA synthesis in the fractionated plasma. In keeping with the results of the original assay of heated unfractionated material, stimulatory activity in the heated fractionated material was significant at 5 and 7 d of ozone exposure. The magnitude of the observed response with heated fractionated samples suggests that all of the effect observed in the heated but unfractionated samples could be accounted for by the activity in the selected fractions.

This same material had no significant effect on DNA synthesis by isolated fetal lung fibroblasts (Table 1), used as a negative control and as a test for cell type specificity.

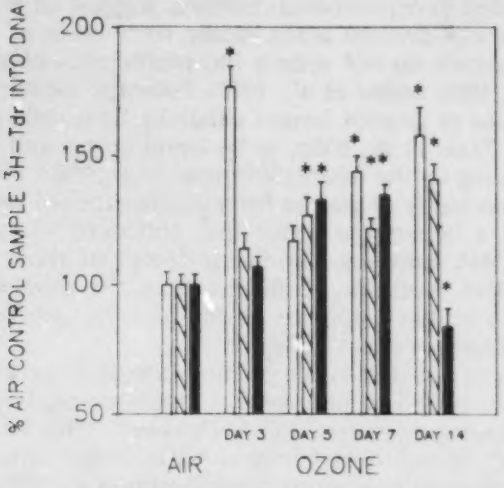

FIGURE 6. Individual plasma samples (mean ± SEM; n = 5–7 for each group) were subjected to sequential fractionation by preparative electrofocusing, then high-performance liquid chromatography, to obtain only those plasma components with an isoelectric point of 6.45–6.75 and apparent molecular weights of 38 ± 3 kDa. This enriched material was restored to the original volume and diluted 10-fold (□) or 5-fold (▨) for assessment of effect on DNA synthesis by cultured lung pneumocytes. In addition 5-fold diluted material was also studied after heat treatment (■). For the purpose of presentation, the incorporation of [^{3}H]thymidine (Tdr) into DNA by each air group has been normalized to a value of 100%, and all other data are expressed relative to air group values; *p <.05 compared with the comparable air group.

DISCUSSION

The biological control mechanisms by which pneumocyte hyperplasia occurs following oxidant injury to the lung have not been established. It is even contentious whether the stimulus to cellular hyperplasia following oxidant injury comes from local events within the lung, or is a result of some systemic response to oxidant inhalation. Experiments of

TABLE 1. Effect of H-I Plasma Fractions with an Isoelectric Point of pH 6.45–6.75 and Apparent Molecular Weight 38 ± 3 kDa on DNA Synthesis by Fetal Lung Fibroblasts

Exposure	Group[a]	N	Mean	SD	SE
Air	3–14	7	100	10	4
Ozone	3	5	94	18	8
	5	5	103	10	4
	7	5	94	23	10
	14	5	103	32	14

[a]Samples were added as 100 μl volume in a total 500 μl volume of culture medium. H-I = heat inactivation at 56°C for 30 min.

nature, duplicated in experimental animals, suggest (at least for O_2) that the stimulus for cell division arises locally, since vascular shunts leading to systemic cyanosis do not reduce the proliferative changes observed (Jentzen et al., 1984; Miller et al., 1970). Potential local mechanisms include the release of growth factors either by lung cells in response to oxidant injury (Tzaki et al., 1988), or by lymphocytes and phagocytes attracted to the lung by the injury (Bitterman et al., 1982; Elias et al., 1985).

Our previous study of plasma from ozone-exposed rats revealed the appearance of a heat-stable factor that enhanced fibroblast, but not pneumocyte, DNA synthesis. The factor described above, which affects pneumocyte DNA synthesis, is distinct from the previously described fibroblast factor in that it appears in different fractions following HPLC separation and has no effect on fibroblasts.

The identity of this heat-stable pneumocyte factor is not yet known. It differs from a 20- to 26-kDa pneumocyte factor released by cultured lung fibroblasts following hyperoxic injury (Tanswell, 1983) both by size and by heat stability. It also differs from a 7-kDa serum pneumocyte factor that appears following pneumonectomy (Smith et al., 1980), and a 30-kDa heat-labile platelet-derived growth factorlike factor that appears to be produced by fetal lung fibroblasts during lung development (Stiles et al., 1986). Based on its isoelectric point and size, we can exclude some of the recognized growth factors. Its isoelectric point excludes platelet-derived growth factor (Westermark and Wasteson, 1976) and fibroblast growth factor (Gospodarowicz et al., 1982), while its apparent molecular weight excludes endothelial cell mitogen (Maciag et al., 1979), rat epidermal growth factor (Moore, 1978), transforming growth factors (Lawrence, 1985), and alveolar macrophage-derived growth factor (Bitterman et al., 1982).

As with our previous report of plasma factors active against fibroblasts, there are a number of outstanding questions that we have been unable to address with this first round of experiments, due to our having exhausted the samples. We assume that the factor is a protein, but need to confirm this by further characterization. The specificity of the factor is also incompletely characterized, in that we do not know whether it is active against epithelial cells from organs other than the lung. The experimental protocol did not, and could not, allow us to define the source of this factor. The most probable source of the factor is the lung itself, though oxidant injury is associated with nonspecific changes in serum proteins (Glass et al., 1984), which could, in theory, account for the observed results.

For the purpose of acting as a marker of oxidant injury, the source of the factor described above is not important. However, it will be necessary to define whether it is detectable following chronic or intermittent exposures at ozone concentrations closer to those experienced by human populations.

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